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Carbone Isotope Ratio Mass Spectrometry of Endogenous Corticosteroids

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Abstract

Our goal was to investigate the differentiation between natural endogenous and exogenous corticosteroids by using Carbon Isotope Ratio Mass Spectrometry (GC/C/IRMS) to detect cortisol/cortisone administration in athletes. As urinary cortisol concentration is rather low for GC/C/IRMS analysis, we focused on the main corticosteroid metabolites: tetrahydrocortisone (THE, 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione) and tetrahydrocortisol (THF, 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one).
The first two steps used for the sample preparations were the same as for anabolic steroids screening: SPE of urine (10mL) on C_{18} column and enzymatic hydrolysis with β-glucuronidase from *E. coli*. The deconjugated corticosteroids were extracted by solid phase extraction (SPE) on C_{18} column with CH_3CN/H_2O (35:65 v/v) mixture. The corticosteroid extracts were then purified by SPE on silica gel column and eluted with CH_3OH/CH_2Cl_2 (10:90) mixture. We studied two different derivatives: the bismethylenedioxy derivatives and the sodium bismuthate oxydation products. All derivatives afforded good GC separations and we were able to perform GC/C/IRMS analysis of urinary metabolites and synthetic cortisol and cortisone. As in the case for testosterone, we found characteristic differences of $\delta^0/_{00}$ values between natural endogenous metabolites (THE and THF metabolite derivatives) and synthetic cortisol and cortisone. For cortisone we noticed significant differences between a recent fabrication lot and ones obtained previously.

1. INTRODUCTION

Corticosteroid use has been restricted in sport. The IOC bans oral or injection administrations. The detection of xenobiotic corticosteroids in urine can be accomplished by classical analytical methods: immunoassays, GC/MS or LC/MS. However, the abuse of endogenous corticosteroids: cortisol (hydrocortisone) or cortisone is difficult to establish as no suitable detection methods have been proposed. We investigated the use of stable carbon isotope analysis to differentiate endogenous and exogenous corticosteroids. This approach has been successfully used for the detection of administration of related endogenous anabolic steroids in man such as testosterone, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA) [1-7] and for the detection of cortisol administration in horses [8].
In fact, the ^{13}C content of natural endogenous steroids reflects an average of the ^{13}C content provided by diet from all different types of plants and is significantly different from exogenous steroids coming from plant sterols, which use soy by-products as a starting point for chemical synthesis. These differences of the ^{13}C content, expressed as a $^{13}C/^{12}C$ ratio, can

be detected by gas chromatography /combustion/isotope ratio mass spectrometry (GC/C/IRMS) measurements.
The first step of the procedure, before GC/C/IRMS analysis, consists of preparing purified urine extracts for corticosteroids which was performed using solid phase extraction as the main purification procedure. The second step comprises of the derivatisation procedure which suitable for GC and C. At this stage trimethylsilylated derivatives must be avoided as silicon is not volatile and leaves deposits in the combustion interface. Acetylation is suitable for androstane (ol and diols) derivatives but leads to an incomplete reaction for corticosteroids bearing 11β- and/or 17α-hydroxyl groups, on the other hand complete derivatisation would afford numerous extra carbon atoms and would modify notably the original 21-carbon content. For this reason we studied two different types of derivatives: bismethylenedioxy [9,10] and bismuthate oxidation products [11,12].

2. EXPERIMENTAL

2.1 Chemicals, reagents
All solvents and reagents were analytical grade purity. The following steroids were obtained from Sigma (St Quentin Fallavier, France): 11β,17α,21-trihydroxy-preg-4-ene-3,20-dione (cortisol, hydrocortisone) ; 17α,21-dihydroxy-preg-4-ene-3,11,20-trione (cortisone) ; 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione (tetrahydrocortisone, THE) ; 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one (tetrahydrocortisol, THF) ; 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one (allo-tetrahydrocortisol, allo-THF) 3α,11β,17α,20β,21-pentahydroxy-5β-pregnane (β-cortol) ; 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one (β-cortolone) ; 3α,11β-dihydroxy-5β-androstan-17-one (11β-hydroxy-etiocholanolone). Cortisol and/or cortisone were also obtained from Roussel-UCLAF (Romainville, France), Steraloids (Wilton N.H., USA), Aldrich (St Quentin Fallavier, France) and Merck (Paris, France).
Boehringer Mannheim (Meylan, France) provided β-glucuronidase from *Escherichia coli.*
Solid phase extractions were carried out on Bakerbond solid phase extraction columns filled with reversed phase octadecyl (500mg, J.T. Baker 7020-06) or normal phase silica gel (500mg, J.T. Baker 7086-03). BSTFA containing 1% TMCS came from Alltech (Templemars, France).

2.2 Urine samples
Urine samples we ˇ obtained from healthy male and female subjects and collected in our institute.

2.3 Preparation of corticosteroids extract
The steroids were extracted and purified as shown in Figure 1. Urine (10mL) was extracted by solid phase extraction (SPE) on C_{18} column, the steroids (free and conjugated) were eluted with methanol (8mL). Once dried, the extract was dissolved in 2mL of 0.2M phosphate buffer pH7 and 40μL of β-glucuronidase was added as supplied. Enzymatic hydrolysis was allowed to proceed for 1H at 37°C. The deconjugated and free corticosteroids were extracted by solid phase extraction (SPE) on C_{18} column by washing with water (12mL) then 12mL of acetonitrile-water (20:80; v/v) and eluting with 12mL of acetonitrile-water (35:65; v/v). After drying, this crude corticosteroid extract was dissolved in 3mL of dichloromethane-methanol mixture (98:2; v/v) and purified by solid phase extraction (SPE) on silica gel column. After washing with 8mL of dichloromethane-methanol (98:2; v/v), the corticosteroid extract was obtained by eluting with 8mL of dichloromethane-methanol (90:10; v/v) and dried.

2.4 Bismethylenedioxy derivatisation

The dried corticosteroid fraction was redissolved in 200µL of dichloromethane and then treated with 500µL of acidified *p*-formaldehyde which was freshly prepared by stirring a mixture of *p*-formaldehyde (1g), water (3mL) and concentrated hydrochloric acid (3mL) at room temperature until the suspension became homogenous. The resulting mixture was vigorously stirred (Vortex) and then sonicated at room temperature for 30 min. After dilution with 2mL of dichloromethane, the aqueous phase was discarded and the organic phase washed successively with saturated aqueous solution of sodium bicarbonate (2mL) and water (2mL). The organic layer containing the bismethylenedioxy (BMD) derivatives was separated and evaporated to dryness under a nitrogen stream.

2.5 Bismuthate oxydation derivatisation

Acetic acid (0.1mL), water (0.1mL) and 5mg of sodium bismuthate were added to the dried corticosteroid fraction. Oxidation was allowed to proceed for 2 hours in darkness at room temperature. After dilution with 5mL of water, the oxidation products were extracted by solid phase extraction (SPE) on a C_{18} column. Acetic acid was eliminated by washing with water (12mL) and the oxidised corticosteroids were recovered with methanol elution (8mL). The derivatives were subjected to dryness over a nitrogen stream.

2.6 Cortols and cortolones characterisation

The dried corticosteroid extract (§2.3) corresponding to 1mL of urine is treated by 100µL of BSTFA+1%TMCS at 70°C for 20 min. The trimethylsilyl derivatives were then analysed by GC/MS on a DB-XLB column (J&W, 30m x 0.25mm I.D., 0.25µm film thickness). The GC temperature program started at 180°C for 1 min, increased at 10°C/min to 300°C and held for 15 min. The column is then purged at 320°C for 5-min. Mass spectra were obtained by scanning a 70-750 Th range. Component structures were confirmed by comparing urinary and authentic steroid TMS derivative spectra.

2.7 GC/MS/EI analysis

GC/MS/EI analysis were performed on a Hewlett-Packard (HP) 5970B mass selective detector linked to a HP5890 gas chromatograph. Helium (100kPa) was the carrier gas. Samples were dissolved in dichloromethane or methanol (100µL) and injected in splitless mode (1µL, 0.7min.) at 280°C. Mass spectra were obtained by scanning a 40-650 Th range. Component structures were confirmed by comparing urinary and authentic steroid spectra.

BMD derivatives were analysed on a DB-XLB column (J&W, 30m x 0.25mm I.D., 0.25µm film thickness). The GC temperature program started at 150°C for 1 min, increased at 15°C/min to 300°C, held for 20 min, increased again at 20°C/min to 320°C and stayed at the final temperature for 4 min.

Bismuthate oxidised derivatives were analysed on a DB-17HT column (J&W, 30m x 0.25mm I.D., 0.25µm film thickness). The GC separation began at 140°C (1 min) then the column temperature was programmed from 140°C to 230°C at 20°C/min, then at 2°C/min from 230 to 280°C and at 8°C/min from 280 to 320°C which was held for 3 min.

Cortols and cortolones were characterised as trimethylsilyl derivatives by GC/MS/EI.

2.8 Isotopic analysis

GC/C/IRMS analysis were performed on a HP5890 gas chromatograph connected to a laboratory-made combustion furnace (Service Central d'Analyse, CNRS) linked to a Finnigan MAT 252 (Bremen, Germany) isotope ratio mass spectrometer. GC conditions were similarly related to those described in §2.7. We used 2µL splitless injection volumes.

The temperature of the combustion oven was 800°C and the oxidative catalyst was a wire of copper oxide (0.25mm) in a quartz tubing of 0.5mm.
The δ standard notation for expressing carbon isotope ratios is defined as the relative difference in isotope ratio between the sample and an international standard (PDB, Pee Dee Belemnite, fossil calcium carbonate from South Carolina), calculated as:

$$\delta\,^{13}C‰ = \frac{R_{sample} - R_{PDB}}{R_{PDB}} \times 1000$$

Where R_x refers to the $^{13}C/^{12}C$ of the sample or international standard PDB. Each extract was injected twice or three times into the GC/C/IRMS and the replicate measurements were averaged.

3. RESULTS and DISCUSSION

We previously demonstrated that the differentiation between endogenous and exogenous steroids by GC/C/IRMS can be performed on the parent compounds and/or on the specific metabolites [1-5]. Using the same approach, we detected cortisol and cortisone based on the measurements of tetrahydrocortisol and tetrahydrocortisone metabolites, respectively. Moreover, cortisol and cortisone are mainly excreted as glucuronide and sulfate conjugates but in low concentrations in urine. Nevertheless, their main metabolites: tetrahydrocortisol and tetrahydrocortisone are excreted in urine mostly as glucuronide, and are present in a more important concentration to allow perform GC/C/IRMS analysis [14].

First steps for steroid analysis include enzymatic hydrolysis of the conjugates. As we wanted to integrate the corticosteroid survey in a global procedure for all the endogenous steroids detection by GC/C/IRMS, β-glucuronidase enzyme from *Escherichia coli* was more convenient with regards to time and specificity. In addition, β-glucuronidase from *E. coli* was recommended as it afforded less complex extracts (Δ^5 steroids are mainly excreted as sulfate conjugates) and did not lead to artefacts [15,16].

All our different steps including extraction and purification of urinary steroids were evaluated using GC/MS/EI. We previously used bismethylenedioxy derivative as have others by GC/MS [8-10]. Nevertheless, to improve our derivatisation procedure, we used an ultrasonic bath for 30-min after of vigorous stirring by Vortex. Bismethylenedioxy-THF and bismethylenedioxy-THE derivatives obtained by this new step was more efficient and showed good GC results with characteristic mass spectra.
The other derivatisation used a specific oxidation of the C17 chain, leading to the formation of 17-keto androstane derivatives. Thus, tetrahydrocortisol was converted into 11β-hydroxy-etiocholanolone, allo-tetrahydrocortisol into 11β-hydroxy-androsterone and tetrahydrocortisone afforded 11-keto-etiocholanolone. These oxidised derivatives obtained from corticosteroid are also naturally occurring steroid metabolites found in urine and attention must be paid not to mistake these two different origins. The compounds resulting from these transformations were analysed directly by GC/MS without any extra derivatisation. Their chromatographic behaviour was satisfactory to obtain GC/C/IRMS analysis.

As our goal was to develop a new screening method utilising GC/C/IRMS, we used solid phase extraction (SPE) technique for urinary corticosteroid metabolites clean-up. Former works on sample preparation for cortisol analysis by GC/C/IRMS used semi-preparative reversed-phase HPLC. Although this technique is very efficient for sample clean-up, is not practical for batches analysis because it is time consuming.
An overview of our sample preparation is represented on Figure 1. After β-glucuronidase digestion, the hydrolysate was purified by solid phase extraction on C_{18} columns. All the corticosteroids of interest were eluted with 12 ml of H_2O/CH_3CN (65:35; v/v) mixture. The washing fraction H_2O/CH_3CN (80:20; v/v) was checked to confirm that none of the compounds of interest were lost. Two additional and sequential elutions with H_2O/CH_3CN (60:40; v/v) and (40:60; v/v) were used to confirm that all of the compounds were completely eluted. However, this corticosteroid fraction also contained an amount of 11-hydroxy etiocholanolone/androsterone which was also partially eluted with H_2O/CH_3CN (65:35). These androstane derivatives were eliminated using a second SPE purification step on silica gel columns. The less polar compounds (11-hydroxy-androstane metabolites) were first eliminated with CH_2Cl_2/CH_3OH (98:2; v/v) then the more polar compounds (corticosteroids) were eluted with CH_2Cl_2/CH_3OH (90:10; v/v). In order to quantify the recovery, cortisol as a reference standard, was added after the two purification steps on SPE columns (C_{18} and silica gel). The calculated recovery was 90% for THF and better than 90% for THE in a range of 4 to 40 μg using bismethylenedioxy derivatives.

The purification procedure was applied to different blank male and female urines.
The extracts were first derivatised to obtain bismethylenedioxy corticosteroids. We obtained characteristic total ion chromatograms by GC/MS/EI analysis (Figure 2). As expected we observed a stronger signal for THE-BMD derivative than for THF-BMD [14] and a very weak signal for cortisol-BMD. In fact, THE-BMD derivative showed two peaks with broad separation at t_R 21.41 and t_R 21.70 min. Both of the mass spectra were very similar so the former compound was assumed to be the allo-THE-BMD derivative. Monomethylenedioxy derivatives were also characterised at t_R 20.68 and 22.17 min with a molecular ion at m/z 378. They corresponded to the addition of only one molecule of formaldehyde to corticosteroid metabolites related to cortolones. Cortols and cortolones are hexahydro metabolites of cortisol and cortisone respectively, and they represent about 40% of the tetrahydro metabolites [14]. Their presence was confirmed using trimethylsilyl derivatives (TMS) of α-cortolone and β-cortolone as reference standards (Sigma).
GC/C/IRMS measurements of BMD derivatives were performed on 8 urine samples and on a synthetic cortisol reference compound from Aldrich. In some cases, we obtained measurements of the two peaks partially separated and we observed identical δ‰ values. As this separation was often difficult to perform, the values were obtained using a global integration as a single peak. Therefore, the THE-BMD carbon isotope measurements presented in this manuscript (Table 1), in fact, corresponded to the sum of the two THE-BMD isomers (THE + allo-THE). Despite these analytical difficulties, we obtained a significant difference of carbon δ‰ values between natural metabolites and synthetic cortisol (Table 1).

The specific oxidation of urinary corticosteroid metabolites with sodium bismuthate checked by GC/MS/EI, led to a very clear chromatogram (Figure 3), without any further derivatisation., oxidised-THE (11-keto-etiocholanolone) and oxidised-THF (11-hydroxy-etiocholanolone) were then measured by GC/C/IRMS (Table 2).
We obtained values for bismuthate oxydation products which were close to BMD-derivative in spite of 4 additional carbon atoms (2 carbon atoms from formaldehyde and 2 from the pregnane chain). We also analysed synthetic cortisol and cortisone from different providers

using bismuthate oxidation (Table 3). The results for synthetic cortisol were very close: -26 to -28 δ‰, but for cortisone it showed clearly two different origins with -26.7 δ‰ (cortisone Roussel) and about -14 δ‰ from other providers (Table 3). It was probably the result of two very different starting materials for the cortisone synthesis. In any case, the significant differences between natural metabolites and synthetic steroids were relevant.

This study was performed using corticosteroid extracts from 10 ml of urine samples. The relative high concentration of cortisol and cortisone metabolites found in our purified extracts were in accordance with Shackleton's results [14] and allowed us to use less than 5 ml urine sample volumes. The described corticosteroids purification procedure for GC/C/IRMS analysis could be entirely integrated into a general method including androstane and pregnane steroids [16]. In fact, after the corticosteroid metabolites elution with H_2O/CH_3CN (65:35, v/v), a second elution with CH_3CN (100%), was used to recover testosterone metabolites and pregnane steroids. This fraction after acetylation allowed us to detect testosterone and dihydrotestosterone administration by GC/C/IRMS [4,5,16].

The two kinds of derivatives used : (bismethylenedioxy and sodium bismuthate oxidation products) were suitable for GC/C/IRMS analysis of corticosteroid metabolites. Nevertheless, bismuthate oxidised metabolites presented more advantages for GC analysis, because the lower ramp of temperature used, avoids bleeding from the GC column and is more suitable for GC/C/IRMS analysis. We also obtained a simpler chromatogram and a better sensitivity for bismuthate oxidised products, because cortol/cortolone hexahydro metabolites were converted into the same derivatives as tetrahydro metabolites (THE, THF, allo-THF). Moreover, the medium polarity of the GC column (DB17 type) used for oxidised corticosteroid metabolites was recommended for androstane metabolites [4,5].

The significant difference observed in carbon isotope ratio measurements between synthetic and natural corticosteroid made an interesting preliminary study. These results should be confirmed in a more expanded investigation of cortisol/cortisone intake or administration, using a controlled study group. In addition, other oxidative reagent such as PCC (pyridinium chlorochromate) [12,17] that afforded completely oxidised derivatives (androstane-3,11,17-trione) with better chromatographic behaviour must be studied.

References

1. M. Becchi, R. Aguilera, Y. Farizon, M.M. Flament, H. Casabianca and P . James, *Rapid Commun. Mass Spectrom.* 8 (1994) 304-308.
2. R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic and H.G. Pope, *J. Mass Spectrom.,* 31 (1996) 169-176.
3. R. Aguilera, M. Becchi, C. Grenot, H. Casabianca and C.K. Hatton, *J. Chromatogr. B* 687 (1996) 43-53.
4. C.H.L. Shackleton, A. Phillips, T. Chang and Y. Li, *Steroids,* 62 (1997) 379-387.
5. C.H.L. Shackleton, E. Roitman, A. Phillips, and T. Chang, *Steroids,* 62 (1997) 665-673.
6. S. Horning, H. Geyer, M. Machnik, W. Schänzer, A. Hilkert and J. Oeβelmann, *Recent Adv. Doping Anal.(4) – Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, Sport und Buch Strauβ (1997) 275-283.
7. S. Horning, H. Geyer, U. Flenker and W. Schänzer, *Recent Adv. Doping Anal.(5) - Proceedings of the 15th Cologne Workshop on Dope Analysis 1997*, Sport und Buch

Strauβ, (1998) 135-148.
8. R. Aguilera, M. Becchi, L. Mateus, M.A. Popot, Y. Bonnaire, H. Casabianca and C.K. Hatton, *J. Chromatogr. B,* 702 (1997) 85-91.
9. H. Shibasaki, T. Furuta and Y. Kasuya, *J. Chromatog.,* 579 (1992) 193-202.
10. P. Varelis, G.A. Smythe, D. Hogson, and L. Lazarus, *J. Chromatog. B,* 660 (1994) 151-157.
11. I.E. Bush, *The Chromatography of Steroids,* (1961) 358-371.
12. E.P. Oliveto, *Organic Reactions in Steroid Chemistry,* 2 (1972) 127-236.
13. M. Palermo, C. Gomez-Sanchez, E. Roitman and C.H.L. Shackleton, *Steroids,* 61 (1996) 583-589.
14. C.H.L. Shackleton, *J. Steroid Biochem. Molec. Biol.,* 45 (1993) 127-140.
15. R. Masse, C. Ayotte and R. Dugal, *J. Chromatogr.,* 489 (1989) 23-50.
16. R. Aguilera, D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R. Swerdloff, H.G. Pope and C.K. Hatton, *J. Chromatogr. B,* 727 (1999) 95-105.
17. K. Kayganish, J.T. Watson, C. Kits and J. Richie, *Biomed. Environ. Mass Spectrom.,* 19 (1990) 341-347.

Figure 1 - Sample preparation flow chart

10 mL urine sample

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Solid phase extaction C18

Washing : H_2O

Elution : CH_3OH

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Deconjugation

β-glucuronidase *E. coli* (1hour at 37°C)

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SPE C18

1st Washing : H_2O

2nd Washing : H_2O / CH_3CN (80:20, v/v)

Elution : H_2O / CH_3CN (65:35, v/v)

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SPE Silica gel

Introduction : dry extract + 3mL CH_2Cl_2 / CH_3OH (98:2, v/v)

Washing : CH_2Cl_2 / CH_3OH (98:2, v/v)

Elution : CH_2Cl_2 / CH_3OH (90:10, v/v)

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Corticosteroids extract

Table 1- Isotopic measurements of Bismethylenedioxy derivatives of Corticosteroids.

Urine samples	THE $^{13}C\ \delta^0/_{00}$	THF $^{13}C\ \delta^0/_{00}$
Male 1	-22.1	-22.4
Male 2	-21.5	-21.8
Male 3	-20.8	-21.8
Male 4	-21.6	-23.1
Female 1	-23.2	-23.0
Female 2	-22.3	-23.5
Female 3	-22.3	-23.8
Female 4	-20.5	n.a.
mean	-21.8	-22.7
C.V.	0.8	0.75
Chemicals	CORTISOL $^{13}C\ \delta^0/_{00}$	
Cortisol from ALDRICH	-30.7	

Table 2 - Isotopic measurements of urinary Corticosteroids using $NaBiO_3$ oxidation

Urine samples	Oxidized-THE $^{13}C\ \delta^0/_{00}$	Oxidized-THF $^{13}C\ \delta^0/_{00}$
Male 1	-22.0	-23.0
Male 2	-20.6	-22.3
Female 1	-20.8	-21.9
Female 2	-21.8	n.a.
mean	-21.3	-22.4
C.V.	0.7	0.6

Table 3 - Isotopic measurements of Cortisol and Cortisone chemicals using $NaBiO_3$ oxidation

Chemical samples	$^{13}C\ \delta^0/_{00}$
Oxidized-CORTISOL	
ROUSSEL	-26.0
SIGMA	-27.8
ALDRICH	-28.0
STERALOIDS	-27.2
Oxidized-CORTISONE	
ROUSSEL	-26.7
SIGMA	-14.3
STERALOIDS	-13.4
MERCK	-14.7